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AD_____

Award Number: DAMD17-01-1-0610

TITLE: Regulation of the EphA2 Tyrosine Phosphorylation by the Human Cytoplasmic Protein Tyrosine Phosphatase

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REPORT DATE: June 2002

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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Carole B. Christian _____

8/5/02 _____

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	June 2002	Final (1 Jun 01 - 31 May 02)

4. TITLE AND SUBTITLE
Regulation of the EphA2 Tyrosine Phosphorylation by the Human Cytoplasmic Protein Tyrosine Phosphatase

5. FUNDING NUMBERS
DAMD17-01-1-0610

6. AUTHOR(S)
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8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution authorized to U.S. Government agencies only (proprietary information, Jun 02). Other requests for this document shall be referred to U.S. Army Medical Research and Materiel Command, 504 Scott Street, Fort Detrick, Maryland 21702-5012.

12b. DISTRIBUTION CODE

13. ABSTRACT ***(Maximum 200 Words)***

Intracellular signaling by protein tyrosine phosphorylation is generally understood to govern many aspects of cellular behavior. The biological consequences of this signaling pathway are important because the levels of protein tyrosine phosphorylation are frequently elevated in cancer cells. In the classic paradigm, tyrosine kinases promote tumor cell growth, survival and invasiveness whereas tyrosine phosphatases negatively regulate these same behaviors. Here, we identify one particular tyrosine phosphatase, LMW-PTP, which is frequently overexpressed in transformed cells. We also show that overexpression of LMW-PTP is sufficient to confer malignant transformation upon non-transformed mammary epithelial cells. Notably, we show that the EphA2 receptor tyrosine kinase is a prominent substrate for LMW-PTP and that the oncogenic activities of LMW-PTP result from altered EphA2 expression and function. These results suggest a role for LMW-PTP in breast cancer progression and link its oncogenic potential to EphA2.

14. SUBJECT TERMS
breast cancer, EphA2, tyrosine phosphatase, LMW-PTP, HCPTP

15. NUMBER OF PAGES
33

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

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INTRODUCTION:

Cancer arises when a population of cells gains the ability to inappropriately grow and survive. These biological behaviors often result from genetic and environmental abnormalities that work together to trigger specific signaling pathways that promote inappropriate cell growth and survival. In particular, increased levels of protein tyrosine phosphorylation are understood to initiate powerful signals that govern many different aspects of cell behavior[1]. A popular paradigm suggests that a balance between tyrosine kinase and phosphatase activities determines the cellular levels of protein tyrosine phosphorylation and thereby governs cellular decisions regarding growth, survival and invasiveness[2]. This model predicts that tyrosine kinases are oncogenic whereas tyrosine phosphatases negatively regulate transformation. Although this paradigm has generally been supported by the identification of oncogenic tyrosine kinases, emerging evidence reveals a more complex interplay between tyrosine kinases and phosphatases. For example, the PTP(CAAX) tyrosine phosphatase has been recently implicated as an oncogene[3]. Moreover, the enzymatic activity of Src family kinases is liberated by phosphatase-mediated dephosphorylation of critical tyrosine residues[4,5]. In the latter situation, phosphatases can actually up-regulate protein tyrosine phosphorylation by increasing the enzymatic activity of kinases.

The EphA2 receptor tyrosine kinase is overexpressed in a large number of human cancers. High levels of EphA2 apply to a large number of different cancers, including breast, prostate, colon and lung carcinomas as well as metastatic melanomas[6-10]. The highest levels of EphA2 are consistently found on the most aggressive cell models of human cancer[9-11]. Moreover, EphA2 is not simply a marker of transformed disease as ectopic overexpression of EphA2 confers tumorigenic and metastatic potential upon non-transformed epithelial cells[10].

In addition to its overexpression, EphA2 is functionally altered in transformed cells as compared with non-transformed epithelia[12]. In particular, EphA2 is prominently tyrosine phosphorylated in non-transformed epithelial cells. Yet, despite its overexpression, the EphA2 in transformed cells is not tyrosine phosphorylated[10-12]. Recent studies indicate that these differences in EphA2 phosphotyrosine content are important because tyrosine phosphorylation of EphA2 causes it to interact with

downstream signaling components which function to negatively regulate cell growth and invasiveness[10,12-16]. In contrast, unphosphorylated EphA2 appears to adopt a different subcellular localization and interacts with different substrates[10-12]. Most importantly, recent studies have shown that unphosphorylated EphA2 functions as a powerful oncoprotein whereas restoration of EphA2 phosphotyrosine content is sufficient to reverse the oncogenic potential of EphA2[10,12,13]. Thus, we were interested in identifying tyrosine phosphatases that might regulate EphA2.

BODY:

Generation of Mutant EphA2:

Based on the differential behaviors of EphA2 in normal and transformed cells, we proposed to investigate the regulation of EphA2 phosphotyrosine content. Our studies investigated the possibility, which is that the phosphotyrosine content of EphA2 in transformed cells is negatively regulated by an associated phosphatase. We affirmed this hypothesis and identified the human cytoplasmic protein tyrosine phosphatase (also known as LMW-PTP) as a phosphatase that regulates EphA2. Please see the attached manuscript (Kikawa *et al.,* Submitted) for a detailed analysis.

Our initial experiment demonstrated that LMW-PTP is overexpressed in many transformed human mammary epithelial cell lines and in tumor-derived breast cancer cells. LMW-PTP has been studies extensively elsewhere but the link with cancer had not yet been established[17-23]. Notably, we found that the highest levels of LMW-PTP were consistently found in cells where EphA2 was not tyrosine phosphorylated.

We then conducted a series of experiments to ask if LMW-PTP might regulate EphA2. Indeed, studies with immunoprecipitated and purified LMW-PTP suggested that LMW-PTP formed an intracellular complex with EphA2 and that EphA2 could serve as a substrate for LMW-PTP.

Based on evidence that dephosphorylation of EphA2 relates to a malignant phenotype, we overexpressed LMW-PTP in a non-transformed human mammary epithelial cells (MCF-10A) and found that overexpression of LMW-PTP was sufficient to confer malignant transformation as defined using a variety of *in vitro* analyses. Interestingly, one consequence of LMW-PTP-mediated transformation was increased

expression of EphA2. Thus, these data related the oncogenic properties of LMW-PTP to EphA2.

Finally, we determined that the oncogenic activities of LMW-PTP required EphA2 overexpression. These studies were conducted using EphA2 antisense oligonucleotides, which selectively inhibit EphA2 expression. This treatment was sufficient to decrease the malignant character of LMW-PTP transformed MCF-10A cells, which indicates that the oncogenic activities of LMW-PTP require EphA2.

KEY RESEARCH ACCOMPLISHMENTS:

- Identification that LMW-PTP is overexpressed in malignant cells
- Determination that overexpression of LMW-PTP is sufficient to induce malignant transformation
- Determination that LMW-PTP interacts with EphA2
- Determination that EphA2 is a substrate for LMW-PTP
- Determination that LMW-PTP overexpression causes EphA2 overexpression
- Determination that LMW-PTP-mediated malignant transformation requires EphA2 overexpression

REPORTABLE OUTCOMES:

- Development of a human mammary epithelial cell line that has been transformed by overexpression of LMW-PTP
- Disclosure and patent (pending) that identify LMW-PTP as a potential target for breast cancer
- Manuscript (in preparation; see Appendix)

CONCLUSIONS:

These studies link overexpression of the LMW-PTP (human cytoplasmic tyrosine phosphatase) with breast cancer. Specifically, high levels of LMW-PTP are found in the most aggressive breast cancer cells and LMW-PTP overexpression is sufficient to confer malignant transformation upon non-transformed human mammary epithelial cells.

Moreover, our studies link the oncogenic properties of LMW-PTP to the regulation of EphA2 tyrosine phosphorylation and overexpression and thus may provide a new target for future breast cancer therapies.

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APPENDICES:

Kikawa *et al.*, manuscript in preparation.

APPENDIX

Regulation of the EphA2 Kinase by the Low Molecular Weight Tyrosine Phosphatase Induces Transformation

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ABSTRACT

Intracellular signaling by protein tyrosine phosphorylation is generally understood to govern many aspects of cellular behavior. The biological consequences of this signaling pathway are important because the levels of protein tyrosine phosphorylation are frequently elevated in cancer cells. In the classic paradigm, tyrosine kinases promote tumor cell growth, survival and invasiveness whereas tyrosine phosphatases negatively regulate these same behaviors. Here, we identify one particular tyrosine phosphatase, LMW-PTP, which is frequently overexpressed in transformed cells. We also show that overexpression of LMW-PTP is sufficient to confer transformation upon non-transformed epithelial cells. Notably, we show that the EphA2 receptor tyrosine kinase is a prominent substrate for LMW-PTP and that the oncogenic activities of LMW-PTP result from altered EphA2 expression and function. These results suggest a role for LMW-PTP in transformed progression and link its oncogenic potential to EphA2.

INTRODUCTION

Cancer arises when a population of cells gains the ability to inappropriately grow and survive. These biological behaviors often result from genetic and environmental abnormalities that work together to trigger specific signaling pathways that promote inappropriate cell growth and survival. In particular, increased levels of protein tyrosine phosphorylation are understood to initiate powerful signals that govern many different aspects of cell behavior (1). A popular paradigm suggests that a balance between tyrosine kinase and phosphatase activities determines the cellular levels of protein tyrosine phosphorylation and thereby governs cellular decisions regarding growth, survival and invasiveness (2). This model predicts that tyrosine kinases are oncogenic whereas tyrosine phosphatases negatively regulate transformation. Although this paradigm has generally been supported by the identification of oncogenic tyrosine kinases, emerging evidence reveals a more complex interplay between tyrosine kinases and phosphatases. For example, the PTP(CAAX) tyrosine phosphatase has been recently implicated as an oncogene (3). Moreover, the enzymatic activity of Src family kinases is liberated by phosphatase-mediated dephosphorylation of critical tyrosine residues (4, 5). In the latter

situation, phosphatases can actually up-regulate protein tyrosine phosphorylation by increasing the enzymatic activity of kinases.

The EphA2 receptor tyrosine kinase is overexpressed in a large number of human cancers. High levels of EphA2 apply to a large number of different cancers, including breast, prostate, colon and lung carcinomas as well as metastatic melanomas (6-10). The highest levels of EphA2 are consistently found on the most aggressive cell models of human cancer (9-11). Moreover, EphA2 is not simply a marker of transformed disease as ectopic overexpression of EphA2 confers tumorigenic and metastatic potential upon non-transformed epithelial cells (10).

In addition to its overexpression, EphA2 is functionally altered in transformed cells as compared with non-transformed epithelia (12). In particular, EphA2 is prominently tyrosine phosphorylated in non-transformed epithelial cells. Yet, despite its overexpression, the EphA2 in transformed cells is not tyrosine phosphorylated (10-12). Recent studies indicate that these differences in EphA2 phosphotyrosine content are important because tyrosine phosphorylation of EphA2 causes it to interact with downstream signaling components which function to negatively regulate cell growth and invasiveness (10, 12-16). In contrast, unphosphorylated EphA2 appears to adopt a different subcellular localization and interacts with different substrates (10-12). Most importantly, recent studies have shown that unphosphorylated EphA2 functions as a powerful oncoprotein whereas restoration of EphA2 phosphotyrosine content is sufficient to reverse the oncogenic potential of EphA2 (10, 12, 13).

Based on the differential behaviors of EphA2 in normal and transformed cells, our laboratory has been investigating the regulation of EphA2 phosphotyrosine content. Our recent studies have centered upon evidence that cancer cells often demonstrate decreased cell-cell contacts (17-19), which destabilize ligand binding (10, 12). Our present studies investigate an additional possibility, which is that the phosphotyrosine content of EphA2 in transformed cells is negatively regulated by an associated phosphatase. We affirm this hypothesis and identify LMW-PTP as a phosphatase that regulates EphA2. Human LMW-PTP has been cloned, sequenced, expressed and structurally characterized (20-22). We also demonstrate that LMW-PTP is overexpressed in many transformed cell lines, and that overexpression of LMW-PTP is sufficient to confer transformation upon non-

transformed epithelial cells. Moreover, we demonstrate that the oncogenic activities of LMW-PTP require EphA2.

MATERIALS AND METHODS:

Cell Lines and Reagents: Human breast (MCF-10Aneo, MCF-10AneoST, MCF-7, MDA-MB-231, MDA-MB-435, SK-BR-3) epithelial cells were cultured as described previously (23, 24), (25). Monoclonal antibodies specific for phospho-tyrosine (PY20) and β-catenin were purchased from Transduction Laboratories (Lexington, KY). Monoclonal antibodies specific for phosphotyrosine (4G10) and EphA2 (clone D7) were purchased from Upstate Biotechnology, Inc. (Lake Placid, NY). Monoclonal antibodies against vinculin were purchased from NeoMarkers (Fremont, CA). Purified LMW-PTP was prepared as described.

Cell Lysates: Cell lysates were harvested and normalized for equal loading as described previously (18). To confirm equal loading, blots were stripped as described previously and reprobed with antibodies specific to β-catenin or vinculin (18).

Immunoprecipitation and Western Blot Analyses: Immunoprecipitation of EphA2 or LMW-PTP were performed using rabbit anti-mouse (Chemicon, Temecula, CA) conjugated Protein A Sepharose (Sigma, St. Louis, MO) as described previously (12). To confirm equal loading, blots were stripped as described previously and reprobed with EphA2 or LMW-PTP specific antibodies (18). Western blot analysis were performed on normalized cells lysates and immunoprecipitations as detailed (12). Antibody binding was detected by enhanced chemiluminescence, (ECL; Pierce, Rockford, IL), and visualized by autoradiography (Kodak X-OMAT; Kodak, Rochester, NY).

EGTA and Pervanadate Treatments: "Calcium Switch" experiments were performed as described previously using MCF-10Aneo cells grown to 70% confluence and medium containing a final concentration of 4mM EGTA (12). Pervanadate was added to MDA-MB-231 in monolayer culture at a final concentration of 0, 1, 10 or 100μM and the treatment was allowed to incubate for 10 minutes at 37°C, 5% CO_2. For the combined EGTA-pervanadate Treatment, MDA-MB-231 cells were first treated with 100μM pervanadate and were then subjected to the EGTA treatment.

In Vitro Kinase and Phosphatase Assays: To evaluate LMW-PTP activity against EphA2, EphA2 was immunoprecipitated from MCF-10Aneo cells and incubated with purified LMW-PTP protein at a concentration of 0.45, 7.8, or 62 μg/mL for 0, 5, 15, or 30 minutes. The assay was terminated through the addition of Laemmli sample buffer. The phosphotyrosine content of the EphA2 in the treatments was then observed using Western blot analysis with antibodies specific to phosphotyrosine. To determine *in vitro* autophosphorylation activity, immunoprecipitated EphA2 was evaluated using *in vitro* kinase assays as detailed previously (12).

Transfection and Selection: Monolayers of MCF-10Aneo cells were grown to 30-50% confluence and were transfected with pcDNA3.1-LMW-PTP or pcDNA3.1-LMW-PTP^{D129A} using Lipofectamine PLUS (Life Technologies, Inc., Grand Island, NY). As a control for the transfection procedure, empty pcDNA3.1 vector was transfected into the same cell line in parallel. Transient transfections were allowed to grow for 48 hours post-transfection. For stable lines, neomycin-resistant cells were selected in growth medium containing 16 μg/mL neomycin (Mediatech, Inc., Herndon, VA). To confirm LMW-PTP overexpression, Western blot analysis was performed using LMW-PTP specific antibodies. Parental cells and cells transfected with empty pcDNA3.1 vector were used as negative controls.

Growth Assay: To evaluate cell growth using monolayer assays, $1x10^5$ cells were seeded into tissue culture treated multi-well dishes for 1, 2, 4 or 6 days in triplicate experiments. Cell numbers were evaluated by trypsin suspension of the samples followed by microscopic evaluation using a hemacytometer. Soft agar colony formation was performed and quantified as detailed (10, 26). For experiments with EphA2 antisense, cells were incubated with oligonucleotides prior to suspension in soft agar. The data shown is representative of at least three different experiments.

Antisense Treatment: Monolayers of MCF-10Aneo cells and MCF-10A cells stably overexpressing LMW-PTP were grown to 30% confluence and were transfected with EphA2 antisense oligonucleotides as detailed. Samples that had been transfected with an inverted EphA2 antisense oligonucleotide or with the transfection reagent alone provided negative controls.

RESULTS:

EphA2 is Regulated by an Associated Tyrosine Phosphatase

Several independent lines of investigation suggested that EphA2 is regulated by an associated tyrosine phosphatase. EphA2 was rapidly dephosphorylated in non-transformed MCF-10Aneo epithelial cells upon disruption of ligand binding. Western blot analysis with phosphotyrosine antibodies (PY20 or 4G10) indicated lower levels of EphA2 phosphotyrosine content within 5 minutes following EGTA treatment, which destabilizes EphA2-ligand binding (12)(Figure 1A). Similarly, tyrosine phosphorylation of EphA2 decreased following incubation of non-transformed epithelial cells with dominant-negative inhibitors of EphA2-ligand binding (e.g., EphA2-F_c, not shown). Similar results were obtained using multiple non-transformed epithelial cell systems, including MCF-12A, MCF10-2, HEK293, MDCK and MDBK cells (not shown). Based on these findings, we asked whether tyrosine phosphatase inhibitors could prevent the loss of EphA2 phosphotyrosine content in response to EGTA treatment. Indeed, inhibitors such as sodium orthovanadate prevented the decrease in EphA2 phosphotyrosine following treatment of MCF-10Aneo cells with EGTA (Figure 1B).

Previous studies by our laboratory have shown that the phosphotyrosine content of EphA2 is greatly reduced in transformed epithelial cells as compared with non-transformed epithelia (10, 12). Thus, we asked if tyrosine phosphatase activity could contribute to the reduced phosphotyrosine content of EphA2 in transformed cells. Although EphA2 was not tyrosine phosphorylated in transformed breast cancer cells (MDA-MB-231, MDA-435, MCFneoST, or PC-3 cells), incubation with increasing concentrations of sodium orthovanadate induced vigorous tyrosine phosphorylation of EphA2 (Figure 1C). As vanadate treatment of cells can often lead to exaggerated phosphorylation of physiologically irrelevant sites, we performed phosphopeptide-mapping studies using EphA2 that had been labeled with ^{32}P-ATP either *in vitro* or *in vivo* (not shown). These studies revealed identical patterns of tyrosine phosphorylation in non-transformed MCF-10Aneo cells and vanadate treated MDA-MB-231 cells (not shown). Although the cytoplasmic domain contains multiple sites that could have been phosphorylated promiscuously, these were not phosphorylated under the conditions utilized here, suggesting that vanadate had not increased the phosphorylation of irrelevant

sites. Altogether, these results indicate that EphA2 is regulated by an associated phosphatase that suppresses EphA2 phosphotyrosine content in transformed cells.

LMW-PTP Interacts with and Dephosphorylates EphA2

To identify tyrosine phosphatases that might regulate EphA2 in transformed cells, we considered a recent report that LMW-PTP regulates a related molecule, EphB4 (27). Our initial experiments began to catalog the expression and function of LMW-PTP in non-transformed (MCF-10Aneo) and transformed (MCF-7, SK-BR-3, MDA-MB-435, MDA-MB-231) mammary epithelial cells (Figure 2). Western blot analyses of whole cell lysates revealed relatively high levels of LMW-PTP in tumor-derived breast cancer cells as compared with non-transformed MCF-10Aneo mammary epithelial cells. The membranes were stripped and re-probed with antibodies against a control protein (vinculin), verifying that the high levels of LMW-PTP did not reflect a loading error or a generalized increase in protein levels in the transformed cells. A transformed variant of MCF-10Aneo, MCFneoST, also demonstrated elevated LMW-PTP expression, which was intriguing based on a recent report that EphA2 is not tyrosine phosphorylated in those cells (11). The use of a genetically-matched system also precluded potential differences due to cell origin or culture conditions. Thus, the highest levels of LMW-PTP were consistently found in transformed epithelial cells and inversely related to EphA2 phosphotyrosine content.

The results suggested that LMW-PTP negatively regulates the phosphotyrosine content of EphA2 in tumor cells. To explore this hypothesis further, we first asked if the two molecules interacted *in vivo*. EphA2 was immunoprecipitated from MDA-MB-231 cells using specific antibodies (clone D7) and these complexes were resolved by SDS-PAGE. Subsequent Western blot analyses revealed that LMW-PTP was prominently found within EphA2 immune complexes (Figure 3A). The inverse experiment confirmed that EphA2 could similarly be detected in complexes of immunoprecipitated LMW-PTP (Figure 3B). Control immunoprecipitations with irrelevant antibodies confirmed the specificity of the interactions of the two molecules (not shown).

The co-immunoprecipitation studies did not clarify whether EphA2 can serve as a substrate for LMW-PTP. To address this directly, EphA2 was immunoprecipitated from

MCF-10Aneo cells, where it is normally tyrosine phosphorylated. The EphA2 immune complexes were then incubated with different concentrations of purified LMW-PTP before Western blot analyses of EphA2 with phosphotyrosine-specific antibodies (PY20 and 4G10). These experiments demonstrated that purified LMW-PTP could dephosphorylate EphA2 in a dose and time-dependent manner (Figure 4).

Although *in vitro* studies indicated that EphA2 could be dephosphorylated by LMW-PTP *in vitro*, we recognized that *in vitro* studies are not always representative of the analogous situation *in vivo*. Thus, LMW-PTP was ectopically overexpressed in MCF-10A cells. This particular cell system was selected because non-transformed MCF-10A cells have low levels of endogenous LMW-PTP and because the EphA2 in these non-transformed epithelial cells is normally tyrosine phosphorylated. Ectopic overexpression of LMW-PTP was achieved by stable transfection, as determined by Western blot analyses with specific antibodies (Figure 5A). Importantly, overexpression of LMW-PTP was sufficient to reduce the phosphotyrosine content of EphA2 as compared with vector-transfected negative controls (Figure 5B). Identical results were obtained using different experiments, with different transfectants and in both stably and transiently-transfected samples (not shown), thus eliminating potential concerns about clonal variation. Moreover, the decreased phosphotyrosine content was specific for EphA2 as the overall phosphotyrosine content of LMW-PTP overexpressing cells was not decreased (Figure 5C).

LMW-PTP Overexpression Causes Transformation of Epithelial Cells

Tyrosine phosphorylated EphA2 negatively regulates tumor cell growth whereas unphosphorylated EphA2 acts as a powerful oncoprotein (10, 12, 13). Thus, we asked whether overexpression of LMW-PTP would be sufficient to induce transformation. To address this question, we utilized the MCF-10A cells, described above, which had been transfected with either wild-type LMW-PTP or a vector control. Our initial studies evaluated the growth rates of control and LMW-PTP-overexpressing cells in monolayer culture. When evaluated using standard, two-dimensional culture conditions, the growth rates of LMW-PTP-overexpressing MCF-10A cells were significantly lower than the growth rates of matched controls ($P<0.05$) (Figure 6A).

Two-dimensional assessments of growth often do not reflect the transformed character of tumor cells. Instead, three-dimensional analyses of cell behavior using soft agar and reconstituted basement membranes can provide a more relevant way of assessing transformed behavior. Whereas vector-transfected MCF-10Aneo cells were largely incapable of colonizing soft agar, LMW-PTP-overexpressing cells formed an average of 4.9 colonies per high-powered microscope field ($P<0.01$; Figure 6B). Based on recent findings with other three-dimensional assay systems, we also evaluated cell behavior using three-dimensional, reconstituted basement membranes. Consistent with a more aggressive phenotype, microscopic assessment of cell behavior in Matrigel confirmed the transformed character of LMW-PTP overexpressing cells (not shown). When plated atop or within Matrigel, LMW-PTP-overexpressing cells formed larger colonies than vector-transfected cells (not shown). Altogether, consistent results with multiple and different systems suggest that overexpression of LMW-PTP is sufficient to induce transformation.

The Transformed Phenotype of LMW-PTP-Overexpressing Cells Requires EphA2

Tyrosine phosphorylation of EphA2 induces its internalization and degradation (13). Thus, we postulated that overexpression of LMW-PTP might increase the protein levels of EphA2. Indeed, Western blot analyses of whole cell lysates revealed higher levels of EphA2 in MCF-10A cells that overexpress LMW-PTP as compared with vector-transfected controls (Figure 5A). Moreover, this EphA2 was not tyrosine phosphorylated (Figure 5B). However, Western blot analyses revealed that the reduced phosphotyrosine content was selective for EphA2, as the general levels of phosphotyrosine were not altered in LMW-PTP transformed cells (Figure 5C).

The finding that overexpression of LMW-PTP increased EphA2 protein levels and decreased its phosphotyrosine content was intriguing since this phenotype was reminiscent of highly aggressive tumor cells (10, 12). Thus, we asked whether selective targeting of LMW-PTP in transformed cells would impact EphA2. To accomplish this, an enzymatic mutant of LMW-PTP^{D129A} that is catalytically inactive (28) was overexpressed in MDA-MB-231 cells, which have high levels of wild-type LMW-PTP (Figure 2) and elevated levels of EphA2 that is hypo-phosphorylated. Ectopic overexpression of LMW-

PTP^{D129A} was found to decrease the levels of EphA2. Moreover, Western blot analyses of immunoprecipitated material demonstrated that EphA2 was tyrosine phosphorylated (Figure 5C). These results indicate that overexpression of wild type LMW-PTP is necessary and sufficient to confer the overexpression and functional alterations of EphA2 that have been observed in tumor-derived cells.

Although the EphA2 in the LMW-PTP overexpressing MCF-10A cells was not tyrosine phosphorylated, it retained enzymatic activity. *In vitro* kinase assays verified that the EphA2 from LMW-PTP-transformed MCF-10A cells had levels of enzymatic activity that were comparable to vector-transfected controls (Figure 7A). To verify equal sample loading, two controls were performed. Equal amounts of input lysate were verified by Western blot analyses with β-catenin antibodies (not shown). In addition, the immunoprecipitated EphA2 was divided and half of the material was resolved by SDS-PAGE and analyzed by Western blot analyses with EphA2 and phosphotyrosine-specific antibodies (Figure 7B and 7C). Thus phosphorylated and unphosphorylated EphA2 were both capable of enzymatic activity.

Having determined that the levels of EphA2 were elevated in LMW-PTP transformed cells, we asked to what extent the oncogenic activity of EphA2 contributed to this phenotype. To address this, we utilized our experience with antisense strategies to selectively decrease EphA2 expression in LMW-PTP transformed cells (13, 29). We verified the success of these strategies by Western blot analyses (Figure 8A) and then asked if decreased EphA2 expression would alter soft agar colonization. Indeed, transfection with EphA2 antisense oligonucleotides decreased the soft agar colonization of LMW-PTP-transformed MCF-10A cells by at least 87% ($P<0.01$; Figures 8B). As a control, transfection of these cells with an inverted antisense nucleotide did not significantly alter soft agar colonization. Thus, we were able to exclude that the results with the antisense oligonucleotides had resulted from non-specific toxicities caused by the transfection procedure. Altogether, our results indicate that the oncogenic actions of overexpressed LMW-PTP require high levels of EphA2.

DISCUSSION:

The major finding of our present study is that EphA2 is regulated by an associated tyrosine phosphatase and we identify LMW-PTP as a critical regulator of EphA2 tyrosine phosphorylation. We also demonstrate that LMW-PTP is overexpressed in metastatic cancer cells and that LMW-PTP overexpression is sufficient to confer transformation upon non-transformed epithelial cells. Finally, we demonstrate that LMW-PTP upregulates the expression of EphA2 and that the oncogenic activities of LMW-PTP require this overexpression of EphA2.

Recent reports from our laboratory and others have shown that many transformed epithelial cells express high levels of EphA2 that is not tyrosine phosphorylated (10, 12). Previously, we had related these depressed levels of EphA2 tyrosine phosphorylation with decreased ligand binding. Transformed cells often have unstable cell-cell contacts and we postulated that this decreases the ability of EphA2 to stably interact with its ligands, which are anchored to the membrane of adjacent cells (12). In part, our present data suggests a new paradigm in which the phosphotyrosine content of EphA2 is also negatively regulated by an associated tyrosine phosphatase that is overexpressed in transformed cells. Given the relationship between EphA2 phosphorylation and cell-cell adhesion, we cannot exclude that cell-cell contacts could also regulate the expression or function of LMW-PTP and future investigation should address this possibility.

The fact that high levels of LMW-PTP were observed in several different cell models of metastatic cancer is notable given that LMW-PTP overexpression is sufficient to confer transformation. LMW-PTP overexpressing cells gain the ability to colonize soft agar and acquire a transformed phenotype when cultured in three-dimensional basement membranes, such as Matrigel. Notably, however, LMW-PTP-overexpressing MCF-10A epithelial cells displayed reduced rates of cell growth as measured using two-dimensional assays of cell growth. This latter observation is consistent with recent reports that high levels of LMW-PTP similarly decrease the monolayer growth rates of other cell types (30, 31). Although such a finding had been interpreted to suggest that LMW-PTP might negatively regulate transformation, our findings support a very different conclusion. Consistent with this, recent studies by our laboratory and others have shown that transformation of MCF-10A cells is often accompanied by decreased monolayer growth

rates and that the most aggressive variants of MCF-10A *in vivo* demonstrate the slowest growth in monolayer culture (10, 11, 32). These findings have implications for the design and interpretation of oncogene function when using non-transformed epithelial cell systems.

The biochemical consequences of EphA2 tyrosine phosphorylation remain largely unclear. Unlike other receptor tyrosine kinases, where autophosphorylation is necessary for enzymatic activity, tyrosine phosphorylation of EphA2 is not required for its enzymatic activity. Consistent with our present results, EphA2 retains comparable levels of enzymatic activity in non-transformed and tumor-derived cells, despite dramatic differences in its phosphotyrosine content (12). Similarly, antibody-mediated stimulation of EphA2 autophosphorylation does not change the levels of EphA2 enzymatic activity (13). Phosphopeptide analyses of the EphA2 cytoplasmic domain provide one potential explanation. Although EphA2 has a predicted activation loop tyrosine at residue 772 (33), neither *in vitro* nor *in vivo* phosphopeptide analyses have identified phosphorylation of this site in normal or malignant cells (M.S. Kinch, unpublished information). Thus, the lack of a consensus activation loop tyrosine may account for the retention of EphA2 enzymatic activity in cells where it is not tyrosine phosphorylated.

Tyrosine phosphorylation of EphA2 does not appear to be necessary for its intrinsic enzymatic activity. Instead, ligand-mediated tyrosine phosphorylation regulates EphA2 protein stability (13). Specifically, tyrosine phosphorylation fates EphA2 to interact with the c-Cbl adapter protein and to subsequently be internalized and degraded within proteosomes (M.S. Kinch and J. Walker-Daniels, Submitted for publication). Consequently, the phosphatase activity of LMW-PTP would be predicted to increase EphA2 protein stability. Indeed, the highest levels of EphA2 are consistently found in cells with high levels of LMW-PTP. One interesting implication of this finding is that it provides a mechanism, independent of genetic regulation of the EphA2 gene, to explain why high levels of EphA2 are found in many different tumors. An alternative possibility is that LMW-PTP upregulates EphA2 gene expression and our present findings do not formally eliminate this possibility. The fact that EphA2 antisense oligonucleotides reversed the transformed character of LMW-PTP overexpressing cells suggests that the

upregulation of EphA2 is relevant to the cellular behaviors of LMW-PTP-mediated transformation.

In summary, our present studies identify LMW-PTP as a new oncogene that is overexpressed in transformed cells. We also link the biochemical and biological actions of overexpressed LMW-PTP with EphA2. These findings have important implications for understanding the biochemical and biological mechanisms that contribute to the metastatic progression of epithelial cells. Moreover, our present studies identify an important signaling system that could ultimately provide an opportunity to target the large number of cancer cells that overexpress EphA2 or LMW-PTP.

Acknowledgments: The authors would like to thank Dr. Al Schroff and the Purdue Cancer Center for producing the monoclonal against LMW-PTP. The authors would also like to thank the current members of Kinch and VanEtten laboratories for their critical reading of the manuscript. This work was supported by award from the NIH (1 U01 CA91318), and the DoD Breast and Prostate Cancer Research Programs.

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FIGURE LEGENDS

Figure 1. EphA2 is regulated by an associated phosphatase. *(A)* Monolayers of MCF-10Aneo human mammary epithelial cells were incubated in the presence or absence (denoted as "C" for control) of 4 mM EGTA for 20 minutes before detergent extraction. The samples were resolved by SDS-PAGE and probed with phosphotyrosine-specific antibodies (PY20 and 4G10; top). The membranes were stripped and reprobed with EphA2 specific antibodies to confirm equal sample loading (below). *(B)* MCF-10Aneo cells were treated with EGTA, as detailed above, in the presence of absence of $NaVO_4$ to inhibit phosphatase activity. *(C)* EphA2 was immunoprecipitated from MDA-MB-231 cells that had been incubated in the presence of the indicated concentrations of $NaVO_4$ for 10 minutes at 37°C.

Figure 2. LMW-PTP protein levels are elevated in transformed cell lines. Detergent lysates (lanes 2-7) were harvested from non-transformed (MCF-10Aneo), oncogene transformed (MCF-10AneoST), and tumor derived (MCF-7, SK-BR-3, MDA-MB-435, MDA-MB-231) mammary epithelial cells. The samples were resolved by SDS-PAGE and subjected to Western Blot analysis using LMW-PTP specific antibodies (top). Purified LMW-PTP (lane 1) provided a positive control for western blot analyses. The membranes were then stripped and reprobed with antibodies specific to vinculin to evaluate sample loading (bottom). Note that LMW-PTP is overexpressed in tumor-derived cells despite the relative over-loading of the non-transformed (MCF-10Aneo) samples.

Figure 3. EphA2 and LMW-PTP form a molecular complex in vivo. *(A)* Complexes of EphA2 were immunoprecipitated from $5x10^6$ MCF-10Aneo or MDA-MB-231 cells, resolved by SDS-PAGE and subjected to Western blot analyses with antibodies specific for LMW-PTP. *(B)* To confirm complex formation, complexes of LMW-PTP were similarly isolated by immunoprecipitation and probed with EphA2 specific antibodies.

Figure 4. EphA2 can serve as a substrate for LMW-PTP in vitro. EphA2 was immunoprecipitated from $5x10^6$ MCF-10Aneo cells before incubation with the indicated amounts of LMW-PTP protein for 0-30 minutes at 37°C. The samples were then resolved by SDS-PAGE and subjected to Western blot analysis with phosphotyrosine-specific antibodies. The membranes were stripped and reprobed with EphA2 specific antibodies to confirm equal sample loading.

Figure 5. LMW-PTP dephosphorylates EphA2 in vivo. *(A-C)* MCF-10A cells were stably transfected with expression vectors that encode for wild-type LMW-PTP. (*A*) Detergent lysates were resolved by SDS-PAGE and subjected to Western blot analyses with LMW-PTP antibodies, with purified LMW-PTP providing a positive control (*top*). Parallel samples were probed for EphA2 protein levels (*middle*). The membranes were stripped and re-probed with β-catenin antibodies as a loading control (*bottom*). *(B)* Equal amounts of EphA2 were isolated by immunoprecipitation, resolved by SDS-PAGE and subjected to Western blot analyses with EphA2 (*top*) or P-Tyr (*bottom*)-specific antibodies. Note that this experiment compared the phosphotyrosine content using equal amounts of EphA2 (to obviate potential concerns about sample loading) as compared

with above *(A)*, which evaluated EphA2 levels in equal amounts of cell lysate. (*C*) Cell lysates were harvested from control or LMW-PTP-transfected cells and equal amounts of protein were resolved by SDS-PAGE and evaluated by Western blot analyses using phosphotyrosine-specific antibodies. *(D)* The protein levels (*top*) and phosphotyrosine content (*bottom*) of immunoprecipitated EphA2 were evaluated using material isolated from MDA-MB-231 cells that had been transfected with a dominant negative form of LMW-PTP^{D129A} or a matched vector control.

Figure 6. LMW-PTP enhances transformed character. *(A)* To evaluate anchorage-dependent cells growth, $1x10^5$ control or LMW-PTP transfected MCF-10A cells were seeded into monolayer culture and cell numbers were evaluated microscopically at the intervals shown. *(B)* In parallel studies, the control and LMW-PTP transfected cells were suspended in soft agar. Shown is colony formation (per high powered field) after five days of incubation at 37°C. These results were representative of at least three separate experiments. * Indicates $p<0.01$

Figure 7. EphA2 retains enzymatic activity in LMW-PTP transformed cells. Equal amounts of EphA2 were immunoprecipitated from control or LMW-PTP transformed MCF-10A cells and subjected to *in vitro* kinase assays. *(A)* Autophosphorylation with γ-^{32}P-labeled ATP was evaluated by autoradiography. To confirm equal sample loading, a portion of the immunoprecipitated materials was evaluated by Western blot analyses with *(B)* EphA2 or *(C)* phosphotyrosine antibodies. Whereas EphA2 is not tyrosine phosphorylated in LMW-PTP transformed cells, it retains enzymatic activity. Note that equal amounts of EphA2 were utilized for these results to overcome differences in endogenous EphA2 expression (for example, See Figure 5B).

Figure 8. Transformation by LMW-PTP requires EphA2 overexpression. LMW-PTP transformed MCF-10A cells were treated with EphA2 antisense (AS oligonucleotides, with inverted antisense (IAS) oligonucleotides or transfection reagents alone providing negative controls. *(A)* Western blot analysis using EphA2 specific antibodies confirmed that the antisense treatment decreased EphA2 protein levels (top). The membrane was then stripped and reprobed for β-catenin to confirm equal sample loading (bottom). *(B)* Parallel samples were suspended and incubated in soft agar for 5 days. Shown are the average number colonies per high-powered microscopic field (HPF). * Indicates $p<0.01$

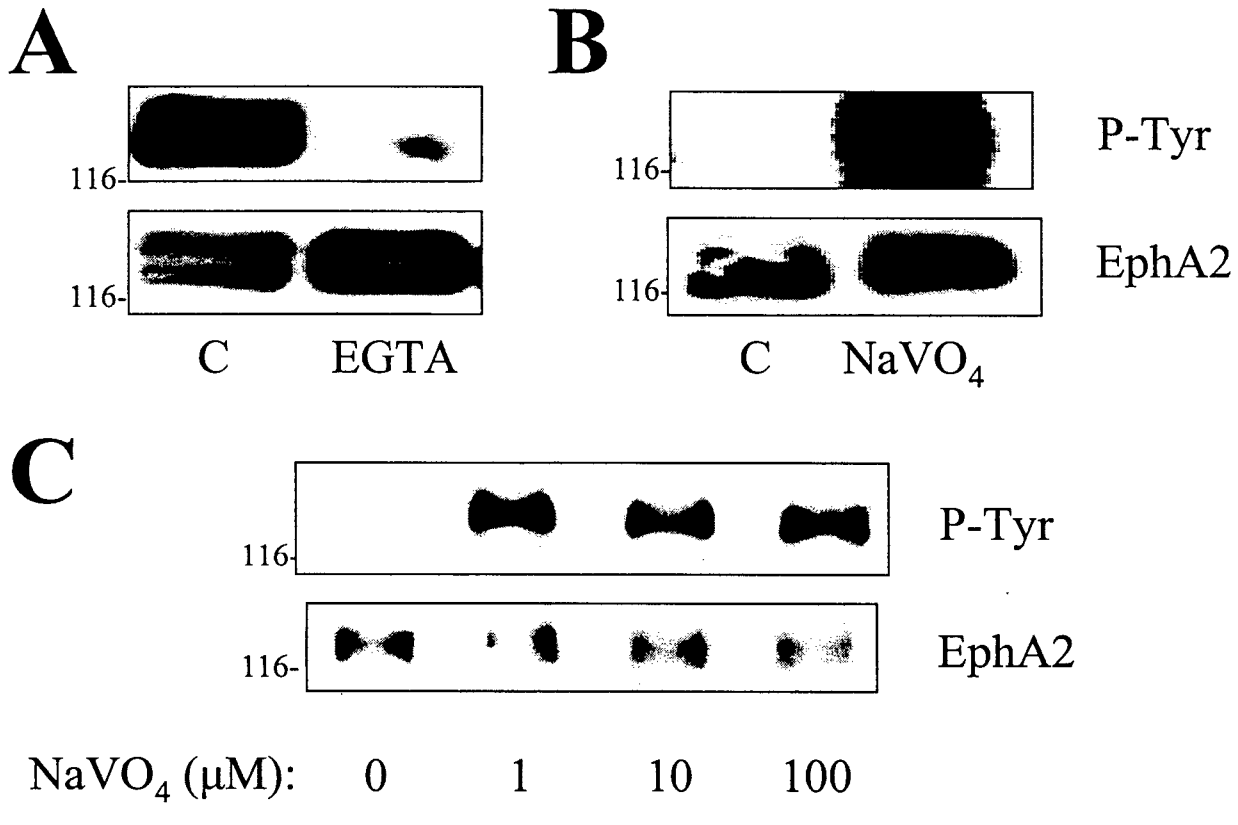

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Figure 1

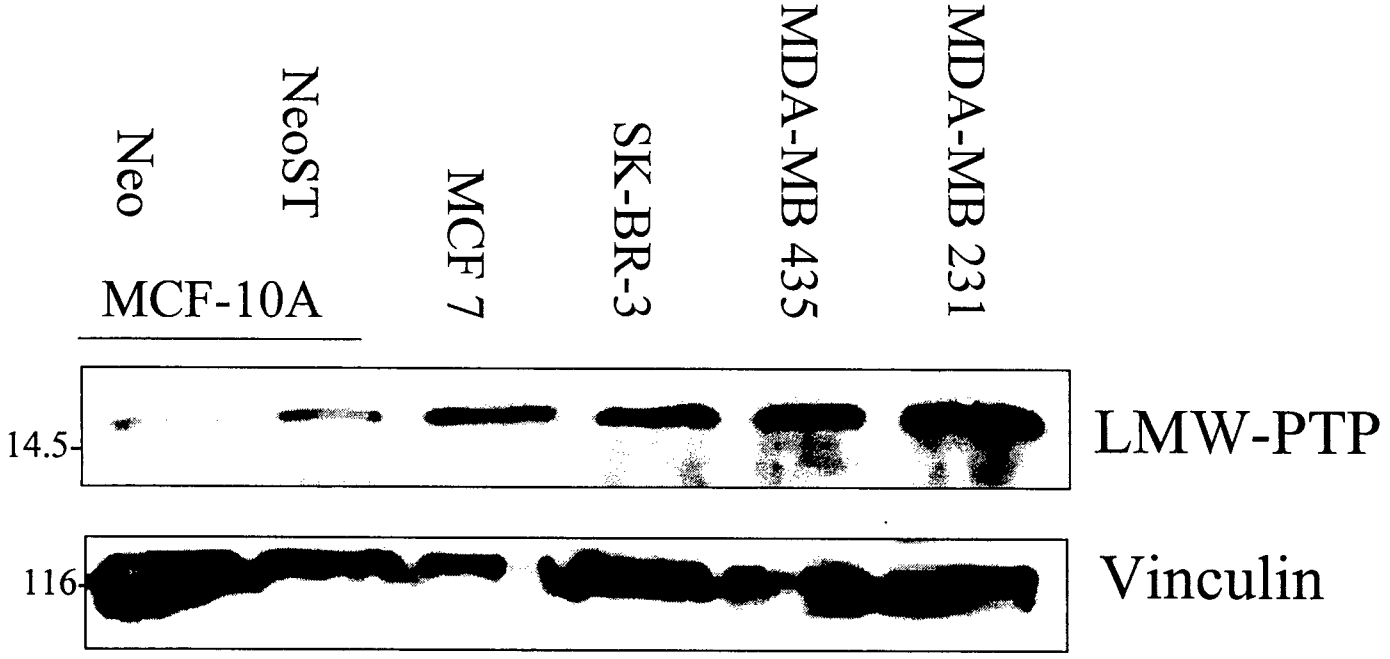

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Figure 2

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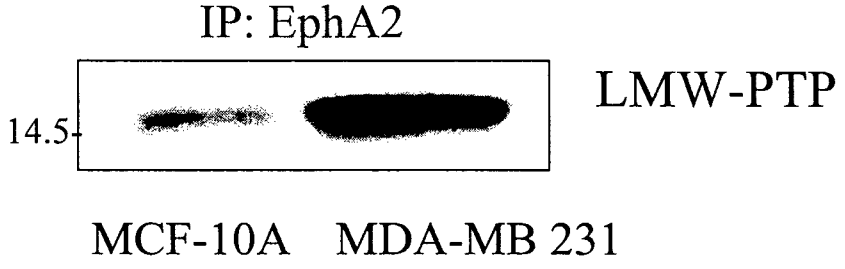

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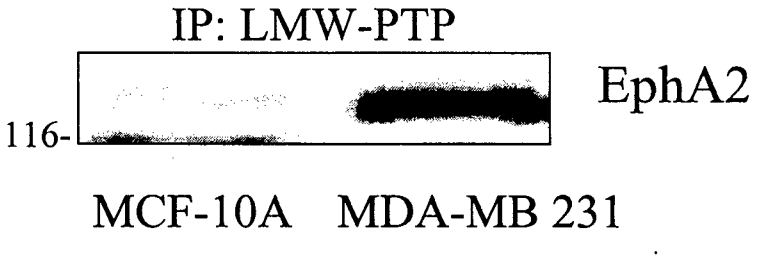

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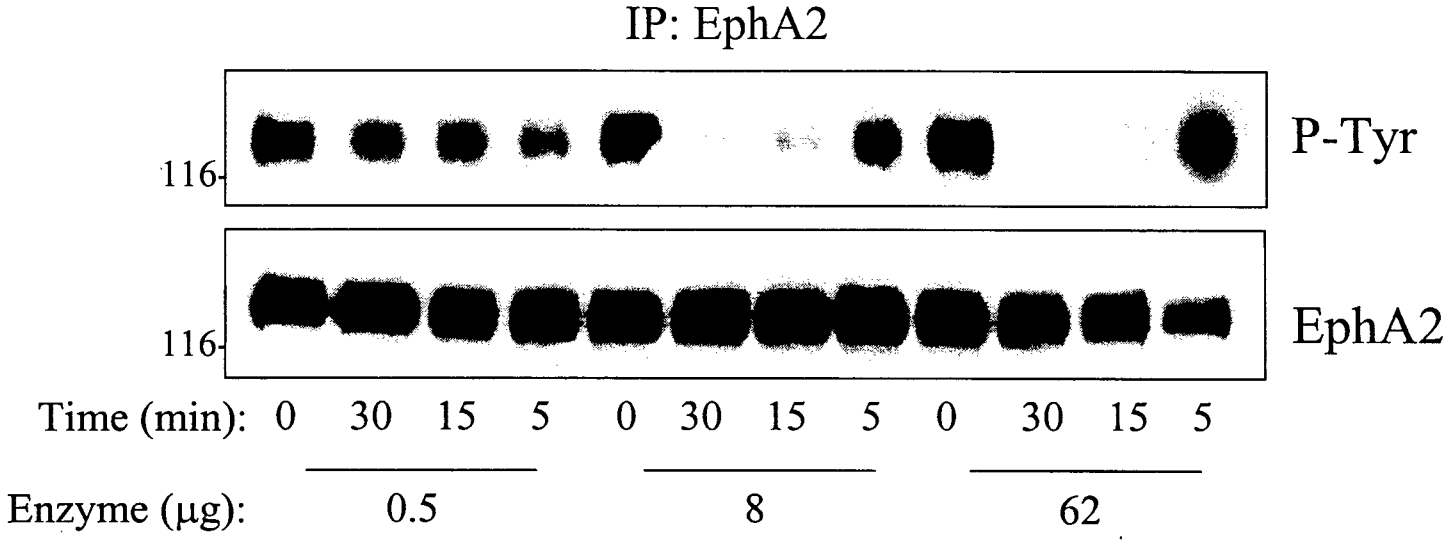

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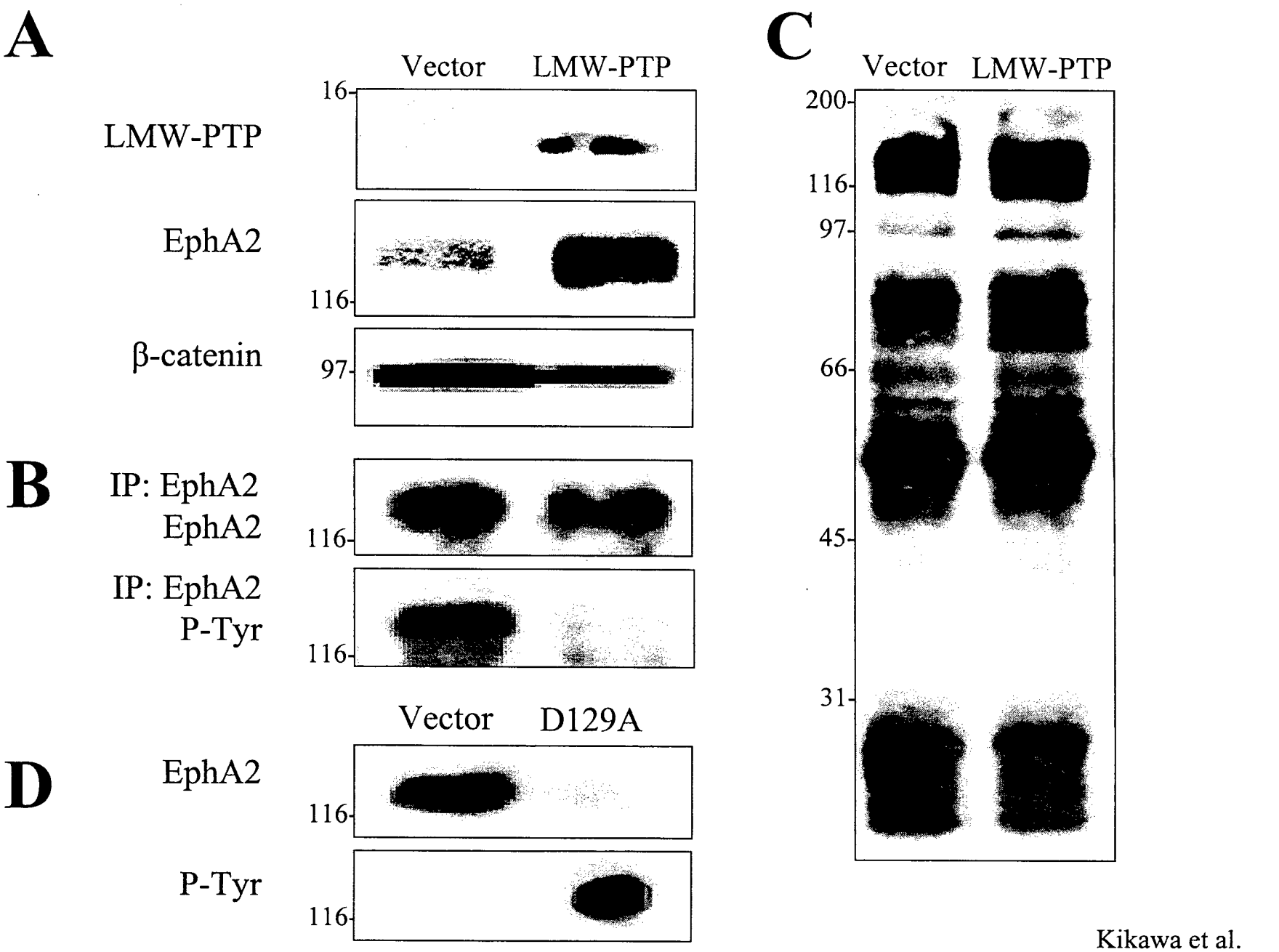

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Figure 5

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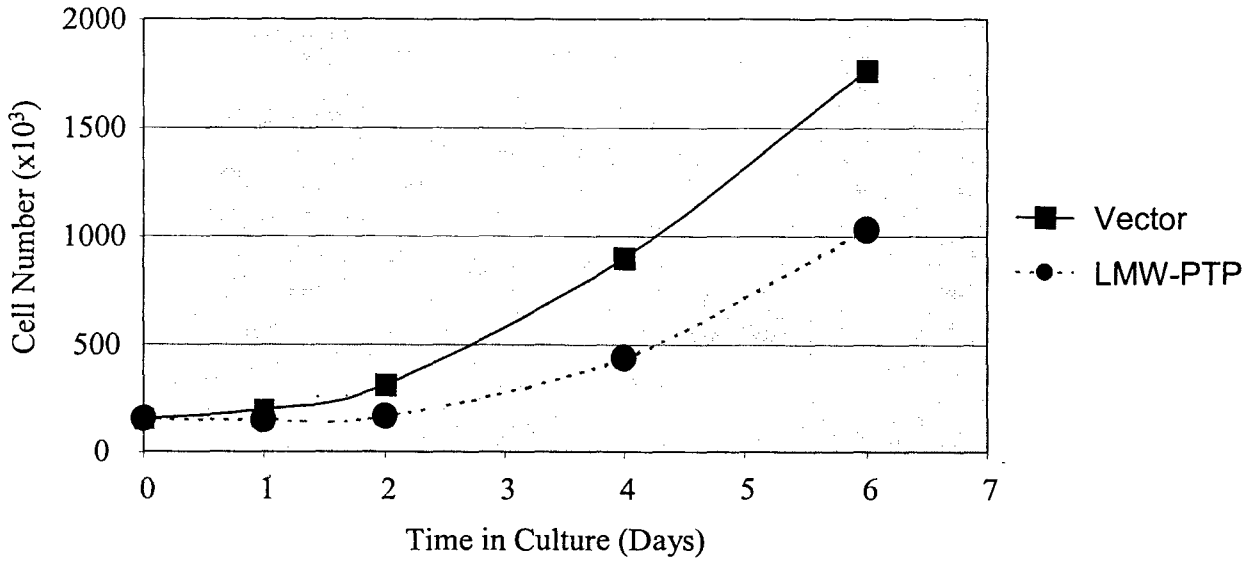

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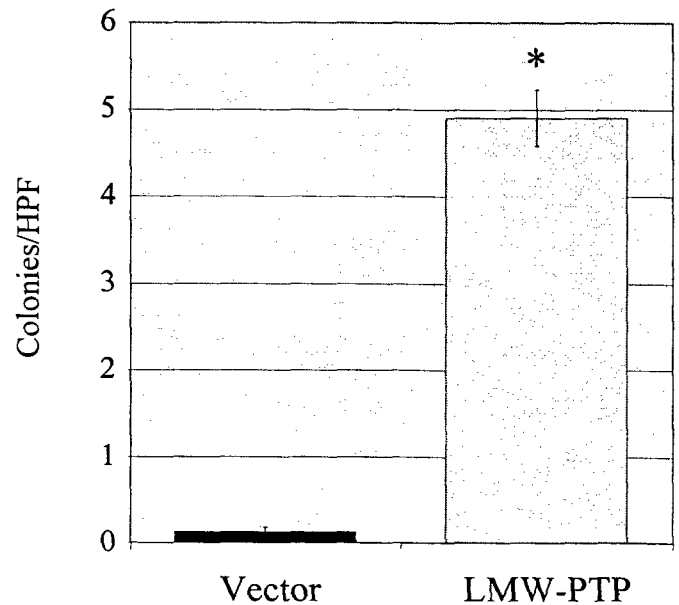

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Figure 6

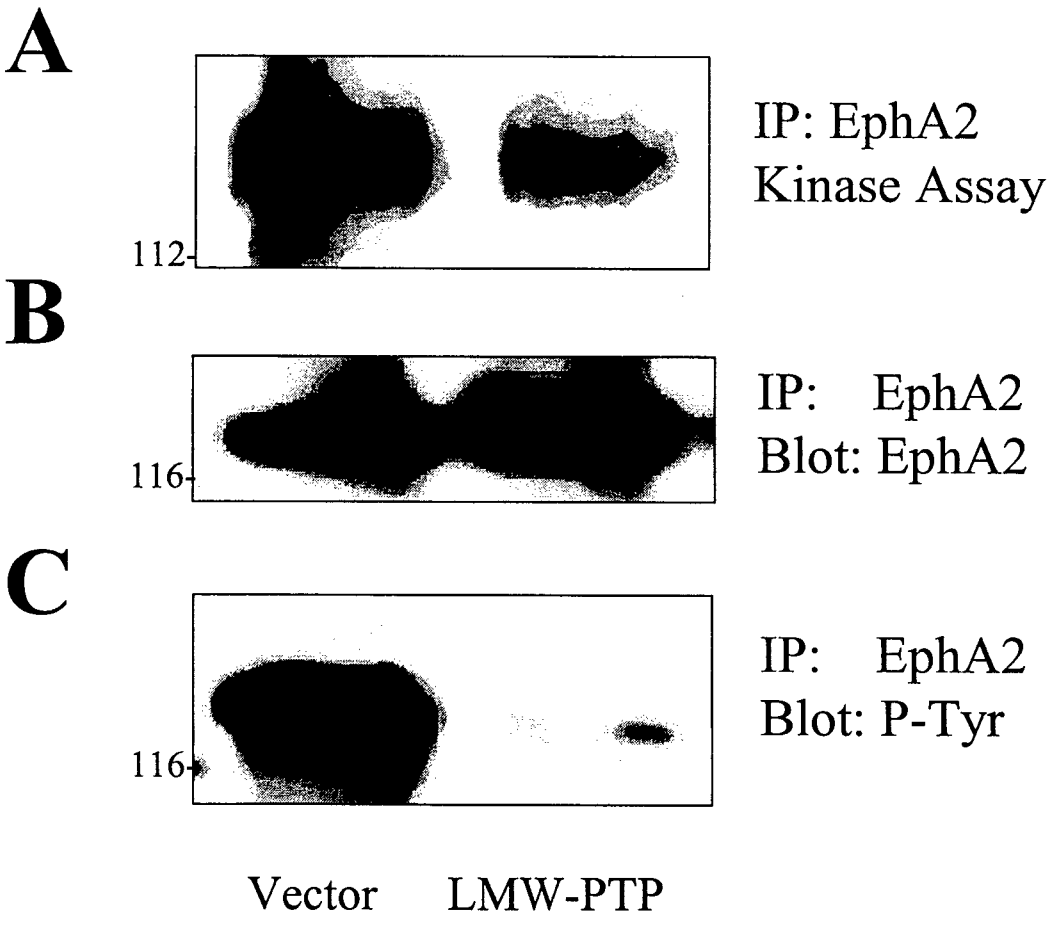

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Figure 7

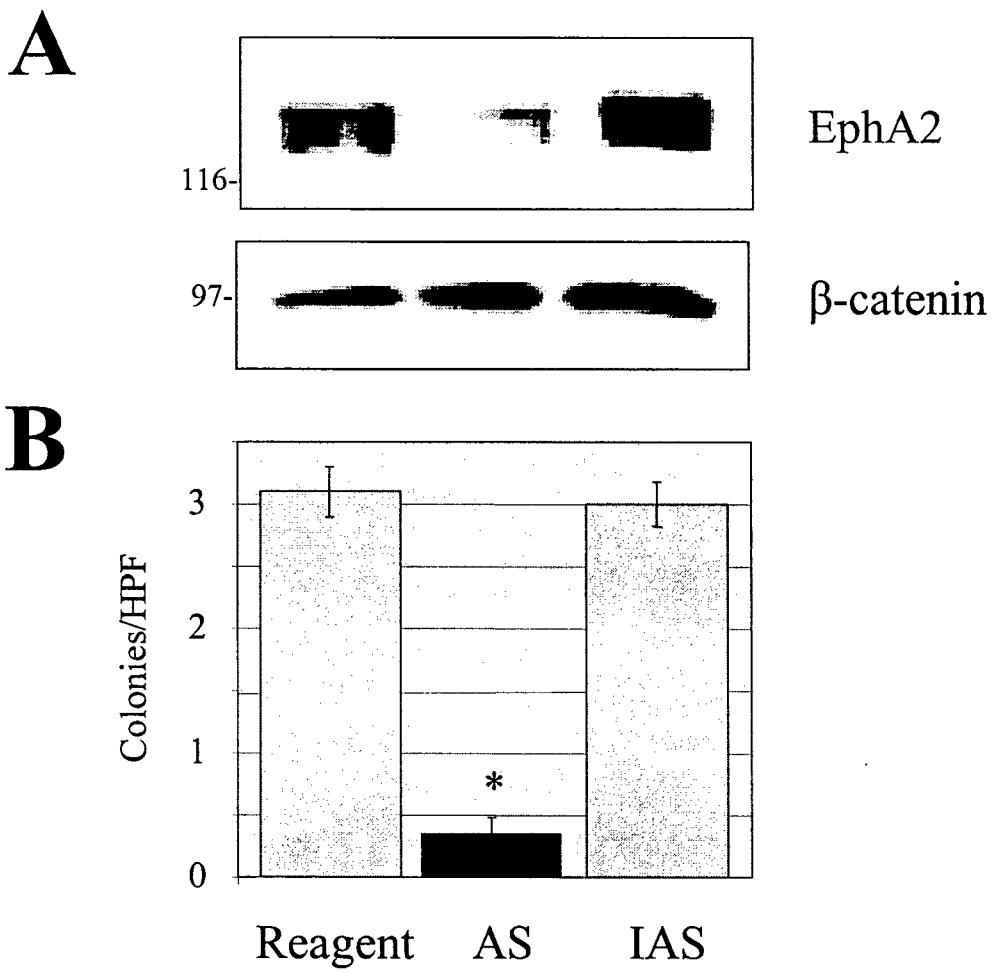

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Figure 8

DEPARTMENT OF THE ARMY
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REPLY TO
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